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HYS2, an essential gene required for DNA replication in *Saccharomyces cerevisiae*

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ABSTRACT

To investigate cell cycle regulation at the S or G2 phase in *Saccharomyces cerevisiae*, we have isolated mutants displaying supersensitivity to hydroxyurea (HU), a chemical that inhibits DNA replication. Such mutants, which we have named hydroxyurea sensitive (*hys*), defined four linkage groups and we characterized the *hys2* mutation in this study. The *hys2-1* mutant displays temperature sensitive growth and a constellation of phenotypes indicating defective DNA metabolism. At the restrictive temperature, *hys2-1* cells arrest as large budded cells with a single nucleus at the neck of the bud and a short spindle. The *hys2-1* mutant exhibits increased rates of chromosome loss and recombination. Additionally, *hys2-1* appears to accumulate incompletely replicated DNA that can be detected by a pulse field electrophoresis assay. Finally, deletion of *RAD9* in a *hys2-1* strain decreases the percentage of arrested cells, suggesting that an intact *RAD9*-checkpoint is required for the cell cycle arrest in *hys2-1* cells. *HYS2* encodes a 55 kDa protein that is essential for viability at all temperatures. Taken together, these data suggest that Hys2 plays a role in DNA replication.

INTRODUCTION

The cell division cycle in eukaryotic cells requires a high degree of coordinated control. The ability to keep order in the pathways that govern and carry out steps of cell division, including DNA replication and mitosis, is thus of fundamental importance in all eukaryotes. Incomplete DNA replication or DNA damage prevents the subsequent mitosis (1,2). In fission yeast, this dependency or checkpoint control has been shown to involve the $p34^{Cdc2}$ kinase. The signal generated by active replication ultimately impinges on the phosphorylation state of Tyr15 of $p34^{Cdc2}$ kinase. This signal transduction pathway is required to prevent mitosis when events in S phase and G2 are perturbed (3,4). In budding yeast, tyrosine phosphorylation of $p34^{Cdc28}$ kinase is not required to arrest mitosis under any of the above circumstances (5,6). Thus, there must be another way of arresting the cell cycle besides tyrosine phosphorylation of $p34^{Cdc28}$. A number of other genes involved in checkpoint control in both yeasts have been identified, although the mechanism that prevents mitosis until completion of DNA replication has not been established (reviewed in refs 1,2,7,8).

DNA replication is fundamental to the maintenance and growth of all eukaryotic cells. *Saccharomyces cerevisiae* provides an excellent model system for identifying the components of the eukaryotic DNA replication machinery because the distinctive growth pattern of this budding yeast allows cell-division-cycle (Cdc) phenotypes to be easily distinguished from defects in other cellular processes (9). For example, studies of *S.cerevisiae cdc* mutants have resulted in the identification of DNA ligase (*CDC9*) (10,11), DNA polymerase α (*CDC17*) (12,13) and DNA polymerase δ (*CDC2*) (14,15). Thus, the determination of the nature of the defect in several *cdc* mutants has led to a better understanding of both DNA replication and mitosis.

Enoch *et al.* (16) have developed a screen for fission yeast mutants defective in coupling mitosis to completion of DNA replication. These mutants have been isolated as hydroxyurea (HU) sensitive mutants that initiate mitosis when DNA replication is blocked by HU. Eight different genes (*rad1*, *rad3*, *rad17*, *hus1*, *hus2*, *hus3*, *hus4* and *hus5*) have been identified, which are required for arrest of mitosis in response to inhibition of DNA replication or to DNA damage. We have searched for mutants that are supersensitive to HU in *S.cerevisiae* to identify genes playing a role in DNA replication itself or in the checkpoint that responds to inhibition of DNA replication. We have isolated four different *hys* mutations (*hys1*, *hys2*, *hys3* and *hys4*).

We report here the characterization of *HYS2*. The *hys2* mutant is HU sensitive and simultaneously confers temperature sensitive growth. The *hys2* mutation causes cells to arrest with a single large bud. The *HYS2* gene is essential for growth and it encodes a 55 kDa protein. The *hys2* mutant exhibits increased levels of mitotic chromosome loss and recombination, suggesting that Hys2 plays a role in DNA replication.

MATERIALS AND METHODS

Strains, media and general methods

Saccharomyces cerevisiae strains used in this study are listed in Table 1. Standard genetic techniques and yeast culture media have been described previously (17,18). Media used to maintain selection for *TRP1* and *URA3* plasmids are synthetic complete media containing 0.5% casamino acid and the appropriate supplements.

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Table 1. List of strains used in this study

Strain	Genotype
KSC688	*MATa ade2 ura1 leu2 lys2*
KSH106	*MATa ade1 his2 trp1 ura3 leu2*
KSH141	*MATa ade2 his3 trp1 ura3*
KSH163	*MATa ade1 his3 trp1 ura3 leu2*
KSH171	*MATα ade1 his2 trp1 ura3 leu2*
KSH172	Isogenic to KSH106 except for *MATa/MATα*
KSH387	*MATa hys3-1 ade1 his2 trp1 ura3 leu2*
KSH389	*MATa hys4-1 ade1 his2 trp1 ura3 leu2*
KSH404	*MATa hys1-1 ade1 his3 trp1 ura3 leu2*
KSH541	*MATα hys2-1 ade1 his2 trp1 ura3 leu2*
KSH542	*MATa hys2-1 ade1 his2 his3 trp1 ura3 leu2*
KSH543	*MATa ade2 his3 trp1 ura3* *MATα ade1 his2 trp1 ura3 leu2*
KSH544	*MATa hys2-1 ade2 his3 trp1 ura3* *MATα hys2-1 ade1 his2 trp1 ura3 leu2*
KSH687	*MATa hys2-1 rad9Δ::LEU2 ade1 his2 his3 trp1 ura3 leu2*
KSH689	*MATa hys2-1 ade2 his3 trp1 ura3*
KSH700	Isogenic to KSH172 except for *HYS2/hys2Δ::LEU2*

DNA was manipulated by standard procedures (19). Sequence analysis was performed by the dideoxy chain termination technique using Sequenase (USB) according to the manufacturer's instruction. Yeast transformation was performed by the alkali cation method (20). One step gene replacement was as described by Rothstein (21).

Mutant isolation

A wild type strain (KSH106) was mutagenized with 2% ethyl methanesulfonate (EMS) to 10% survival. Mutagenized cells were grown to single colonies on YEPD medium at 30°C. Colonies were replica plated to YEPD and YEPD containing 10 mg/ml HU. After 3 or 4 day incubation at 30°C, 19 colonies grew very poorly in the presence of HU. Among them four mutants accumulated >80% large budded cells after a 4 h shift to the HU containing medium.

Cloning of the *HYS2* gene

Strain KSH542 (*hys2-1 ura3*) was transformed with a genomic library constructed on YCp50 at 30°C and then replica plated on YEPD medium containing 10 mg/ml HU. A single plasmid (p12) was identified to complement both the HU and temperature sensitive growth of the *hys2-1* mutation.

To establish that the complementing plasmid p12 carries the *HYS2* gene, we crossed the *hys2-1* mutant with the *hys2Δ::LEU2* (see below) strain carrying plasmid p12. The plasmid p12 was cured from the resulting diploid. Because this diploid is heterozygous for the *hys2-1* mutation, a disruption of the wild type gene should unmask the HU sensitive and temperature sensitive phenotypes if the cloned fragment is exclusively *HYS2* coding sequence. The diploid was HU and temperature sensitive, confirming that the mutation resides within the gene disrupted by the *LEU2* gene. After sporulation and tetrad dissection of diploid, the viability of the spores was assessed. Only half of the spores were viable, and as expected, all of these were HU and temperature sensitive, as well as Leu⁻.

Plasmid construction

The *Sal*I–*Hin*dIII fragment of p12 was subcloned into YCplac22 (22) and YCplac33 (22) creating YCp22-HYS2 and YCp33-HYS2, respectively. This fragment was also cloned into pSP72 (Promega) cleaved with *Sal*I and *Hin*dIII creating pSP-63HS. The nucleotide sequence of the *Sal*I to *Hin*dIII fragment was determined for both strands. For construction of YCpG-HYS2, a 1.5 kb fragment containing the *HYS2* coding sequence was obtained by polymerase chain reaction using the primer GCTCTAGAGGCAATGGACGCATTGT, corresponding to nucleotide residues –4 to +13 of the *HYS2* gene and the primer GCGTCGACTATTTAAATGTCAATCT, corresponding to nucleotide residues +1451 to +1567 of the *HYS2* gene (DDBJ accession no. D50324). *Xba*I and *Sal*I sites included in the oligonucleotides are underlined. The fragment after cleaved with *Xba*I and *Sal*I was cloned into YCpG33 (17), a YCplac33 derivative containing the *GAL1* promoter, creating YCpG-HYS2. The plasmid YCpG-HYS2 complemented a disruption of *HYS2*, even when the cells were grown on YEPD medium.

Disruption of *HYS2*

For construction of the *hys2Δ::LEU2*, plasmid pDhu2L was created by replacing the internal *Bam*HI fragment of *HYS2* with the *LEU2* gene. The *Xba*I-*Pvu*II fragment containing *hys2Δ::LEU2* was integrated into a diploid strain KSH172, creating strain KSH700. After sporulation, tetrad analysis was used to determine the phenotype caused by the deletion.

UV radiation and DNA damaging agent sensitivities

UV radiation at 254 nm was delivered by use of a Stratagene Stratalinker. Cells grown exponentially at 30°C were plated on YEPD and then irradiated by UV according to the manufacture's manual. After incubation at 30°C for 3 days, the number of colonies was counted.

Methyl methanesulfonate (MMS) sensitivity assay was described previously (23). Cells grown exponentially in YEPD medium at 30°C were incubated with 0–0.8% of MMS for 40 min. The incubation was terminated by adding sodium thiosulfate to a final concentration of 5%. Aliquots were plated out on YEPD, followed by incubation at 30°C. After 3 days of incubation, the colony number was counted.

Viability assays

The method of Weinert and Hartwell (24) was used to determine whether the *hys2-1 rad9Δ::LEU2* mutant (KSH687) loses viability when incubated at the restrictive temperature. Cells were grown in YEPD medium at 25°C to mid log phase and they were shifted to the restrictive temperature (37°C). Aliquots of the culture were removed at intervals, sonicated and plated on YEPD plates. After incubation at 25°C for 2 days, cell viability was determined by microscopy; individual microcolonies on the plate were scored as either inviable (<16 cell bodies) or viable (>16 cell bodies).

Immunofluorescence microscopic analysis

Cells were processed for fluorescence and indirect immunofluorescence microscopy as described previously (25). Cells were fixed and stained for DNA with 25 μg/ml 4,6-diamidino-2-phenylindole (DAPI). Microtubule structures were observed following formaldehyde fixation using the anti-tubulin monoclonal antibody TAT-1 and a FITC-conjugated goat anti-mouse antibody as described (26).

DNA flow cytometry analysis

Flow cytometry DNA quantitation was determined as described previously (26). Cells (1×10^6–1×10^7) were collected, washed once with 1 ml of water and resuspended in 0.3 ml of 0.2 M Tris-HCl (pH 7.5). Ethanol was added to a final concentration of 70% of with vigorous agitation and cells were stored at –20°C overnight. After resuspension in 0.3 ml of 0.2 M Tris-HCl (pH 7.5), cells were sonicated briefly and preboiled RNase A (Sigma) was added to a final concentration of 1 mg/ml. Following a 3 h incubation at 37°C, propidium iodine (Sigma) was added (final concentration, 5 μg/ml) and the resulting stained cell suspensions were analyzed using a Becton-Dickinson FACScan.

Analysis of chromosome III missegregation

Quantitative measurement of chromosome III loss and recombination were performed essentially as previously described (27). Strains to be tested were allowed to grow at 30°C on YEPD medium. Mating was initiated by mixing ~1×10^6 cells to be tested with 1×10^6 *MATa* haploid tester (KSC688) in a final volume of 200 μl. After 4 h at 30°C the mating mixture was plated on medium selecting for prototrophic triploids. Wild type *MATα* haploid KSH171 was used as a control to determine mating efficiency. To ensure that the rate of *MATα* homozygosis or chromosome III missegregation was not underestimated due to a mating defect associated with *hys2-1*, a *hys2-1* haploid strain KSH541 was tested. This revealed that no mating deficiency associated with *hys2-1* at 30°C. The combined rate of *MATα* homozygosis plus chromosome III loss was determined from the total number of cells that mated. The rate of chromosome III loss was calculated by subtracting the rate of mitotic recombination determined by analysis of Leu^+ maters from the combined rate. The assay was performed twice using independent colonies.

Pulse field gel analysis

Cultures were grown to early log phase at 25°C and then incubated at 37°C for 4 h. For control samples, cultures of *hys2-1* cells were incubated with α-factor (10 μg/ml) for 2.5 h, HU (10 mg/ml) for 4 h or nocodazole (20 μg/ml) for 4 h at 25°C. Yeast chromosomal DNA samples were prepared as previously described (28). Pulse field gel electrophoresis was carried out in 1% agarose (LE, FMC) in a pulsaphor electrophoresis unit (Pharmacia). Electrophoresis was performed for 24 h with a switching time of 70 s at 170 V in 0.5 × Tris-borate-EDTA (TBE). The DNA was transferred onto nylon membranes and probed with a ^{32}P-labeled *TRP1* fragment (the 1.4 kb *Eco*RI from YRp7 plasmid). This probe to chromosome IV was chosen because this blot analysis is most sensitive when probes derived from large chromosomes are used (28).

Cell synchronization and RNA analysis

Synchronization by α-factor mating pheromone and Northern blot analysis were performed as described previously (29). DNA probes were: *CLN2*, the 0.9 kb *Xho*I-*Hin*dIII fragment of the *CLN2* gene (30); *ACT1*, the 1 kb *Xho*I-*Hin*dIII fragment derived from pYS91 containing *ACT*1 cDNA (unpublished); and *HYS2*, the 1 kb *Bam*HI fragment from YCp33-HYS2.

RESULTS

Isolation of *hys* mutants

The culture of wild type cells in the presence of HU (10 mg/ml) temporarily accumulates cells with large buds and eventually recovers from the HU arrest. We mutagenized wild type cells (strain KSH106) with ethyl methanesulfonate (EMS) and screened for colonies that were sensitive to HU by replica-plating cells onto plates with or without HU and identifying clones that failed to form colonies on HU. Because HU blocks DNA synthesis, mutations defective in DNA metabolism are expected to suffer more severe growth retardation in the presence of HU. Also when incubated with HU, mutations deficient in their ability to monitor impaired DNA synthesis would allow cells to enter into mitosis with defective chromosomes, resulting in lethality. Of ~10 000 EMS-mutagenized cells screened, 19 clones showed HU sensitive phenotype. Among the HU sensitive mutants, four of those that accumulated over 80% large budded cells after a 4 h shift to the HU media were identified by microscopic observation. In crosses to a wild type strain, each mutant was recessive for HU sensitivity and segregated as a single gene mutation. Complementation analysis showed that mutants formed different complementation groups, which we are calling *hys1–hys4* (Table 2). In this paper, we present data characterizing the *hys2* mutation.

The HU-treated cultures of *hys2-1* mutant were subjected to cytological examination. Cells were stained with the DNA specific fluorescence dye DAPI for analysis of cell and nuclear morphology. More than 90% of the large budded *hys2-1* cells, like wild type cells, contained a single nucleus near or at the neck with a short spindle (data not shown). In addition to HU sensitive phenotype, the *hys2-1* mutation cells conferred sensitivity to MMS and temperature sensitive growth defect (Table 2). To test if the *hys2* growth defect at 37°C and HU sensitivity are caused by a single mutation, we analyzed the meiotic products from a diploid strain heterozygous for *hys2-1*. Temperature sensitive growth phenotype always cosegregated with the HU sensitive phenotype in tetrads, indicating that the *hys2-1* mutation determines both phenotypes.

The *hys2-1* mutation is defective in DNA replication

To examine the terminal morphology of strains carrying the *hys2-1* mutation, growth-arrested populations were analyzed microscopically (Fig. 1). When exponentially growing cultures of the *hys2-1* mutant or $HYS2^+$ cells grown at 25°C were shifted to 37°C for 4 h, 80% of *hys2-1* cells arrested with large buds, compared with only 19% of the $HYS2^+$. Thus, *hys2-1* exhibits a marked elevation in the proportion of large budded cells at the restrictive temperature.

Table 2. Properties of *hys* mutants

Genotype[a]	Growth[b]			MMS sensitivity[c]	UV sensitivity[c]	
	30°C	37°C	10 mg/ml HU	0.4%	90 J/m^2	120 J/m^2
HYS	+	+	+	16%	30%	7%
hys1	+	+	–	0.09%	0.2%	NT
hys2	+	–	–	0.06%	17%	5%
hys3	+	+	–	NT	NT	NT
hys4	+	+	–	NT	NT	NT

[a]Strains used here were KSH106 (*HYS*), KSH404 (*hys1*), KSH542 (*hys2*), KSH387 (*hys3*) and KSH389 (*hys4*).
[b]Growth of cells at 30 or 37°C was tested by streaking cells on YEPD plates and incubating at the corresponding temperature for 2 days. Cells were tested for growth on YEPD plates containing 10 mg/ml HU at 30°C for 4 days. +, growth; –, no growth.
[c]DNA damage sensitivities were determined by treating log-phase cultures of cells with 0.4% MMS for 40 min or irradiating with 90 or 120 J/m^2 UV light (254 nm) and percent survival was measured after 3 days incubation at 30°C. NT, not tested.

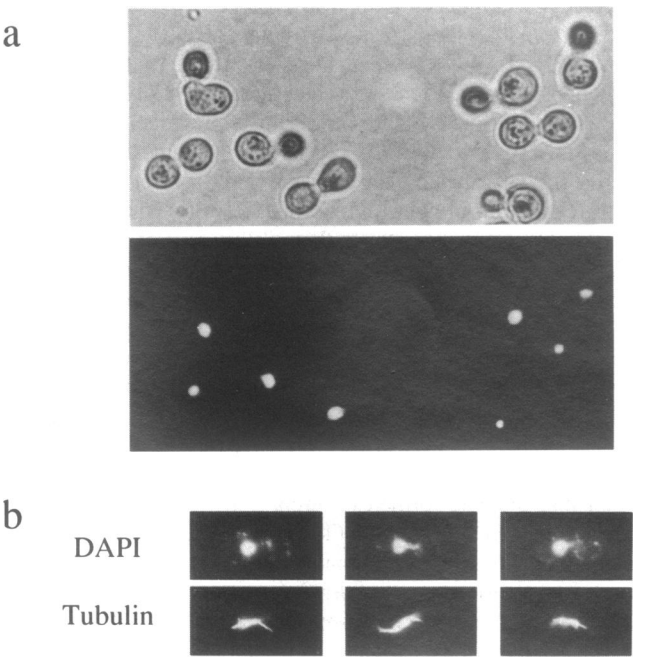

Figure 1. Cell cycle arrest phenotype conferred by the *hys2-1* mutation. (**a**) DAPI stained *hys2–1* cells. A logarithmically growing culture of KSH542 (*hys2-1*) were shifted from 25 to 37°C for 4 h. Cells were fixed in ethanol and examined by phase contrast microscopy (upper panel) for DAPI staining (lower panel). (**b**) Nuclear and microtubule structure of *hys2-1* cells. Cells of KSH542 (*hys2-1*) were grown logarythmically at 25°C and then shifted to 37°C for 4 h. Samples were fixed in formaldehyde and stained with DAPI (upper panel) and anti-tubulin antibodies (lower panel) to visualize nuclei and spindles.

After 4 h incubation at 37°C, 83% of the large budded cells in the *hys2-1* culture had a single undivided nucleus at or through the neck between the mother and daughter cells (Fig. 1a). Following a shift to 37°C, samples of *hys2-1* cells were prepared for anti-tubulin immunofluorescence microscopy (Fig. 1b). The *hys2-1* cells had a short mitotic spindle, consisting of a brightly stained bar of nuclear microtubules with more faintly staining cytoplasmic microtubules. Thus, the phenotype of *hys2-1* mutants at the restrictive temperature is similar to that observed in *cdc* mutants with defects in DNA replication or mitosis (31).

Mutations in genes that are involved in DNA replication, such as those encoding DNA polymerase α (*CDC17*), DNA polymerase δ (*CDC2*) and DNA ligase (*CDC9*), all exhibit significantly elevated frequencies of both genetic recombination and chromosome loss (32). This feature distinguishes them from mitotic defects that show only elevated chromosome loss. We assayed the recombination and chromosome loss frequencies using standard heterozygous markers on chromosome III (see Materials and Methods) (Table 3). Diploids homozygous for the *hys2-1* mutation were grown at the permissive temperature. Frequencies of recombination and chromosome loss were normalized to a *HYS2*$^+$/*HYS2*$^+$ strain. The *hys2-1*/*hys2-1* strain exhibited significant increases in the frequency of both recombination (16-fold increase) and chromosome loss (20-fold increase) even at the permissive temperature (Table 3).

Table 3. Rates of recombination and chromosome III missegregation in the *hys2-1* mutant[a]

Genotype	Recombination rate	Chromosome missegregation rate
HYS2/HYS2	8.6×10^{-5} (1)	9.8×10^{-5} (1)
hys2-1/hys2-1	1.4×10^{-3} (16)	2.0×10^{-3} (20)

[a]The rates with which mating competent diploids arose by mitotic recombination versus chromosome III missegregation in *HYS2/HYS2* (KSH543) and *hys2-1/hys2-1* (KSH544) diploids were analyzed at 30°C. Mating competent diploids were scored as protrophic triploids after incubation with a *leu2* mating tester (KSC688). The fraction of these diploids that had mated due to *MATa* homozygosis by mitotic recombination were distinguished from those that had missegregated chromosome III, by replica plating to plates lacking leucine. Rates were calculated from these numbers as described in Materials and Methods. Numbers in parentheses are normalized to the wild type (*HYS2/HYS2*) background.

To determine the extent of DNA replication in the *hys2-1* mutant when the cells undergo arrest, we examined the DNA content of *hys2-1* cells at the restrictive temperature. We incubated exponentially growing *hys2-1* and *HYS2*$^+$ strains at a permissive (25°C) or restrictive temperature (37°C), stained the cells with propidium iodide and determined the DNA content of the cells by FACS analysis. A high percentage of *hys2-1* cells arrest with a 2N content of DNA at 37°C, indicating that the majority of DNA synthesis can be completed at restrictive temperature (Fig. 2). The informative result from the FACS

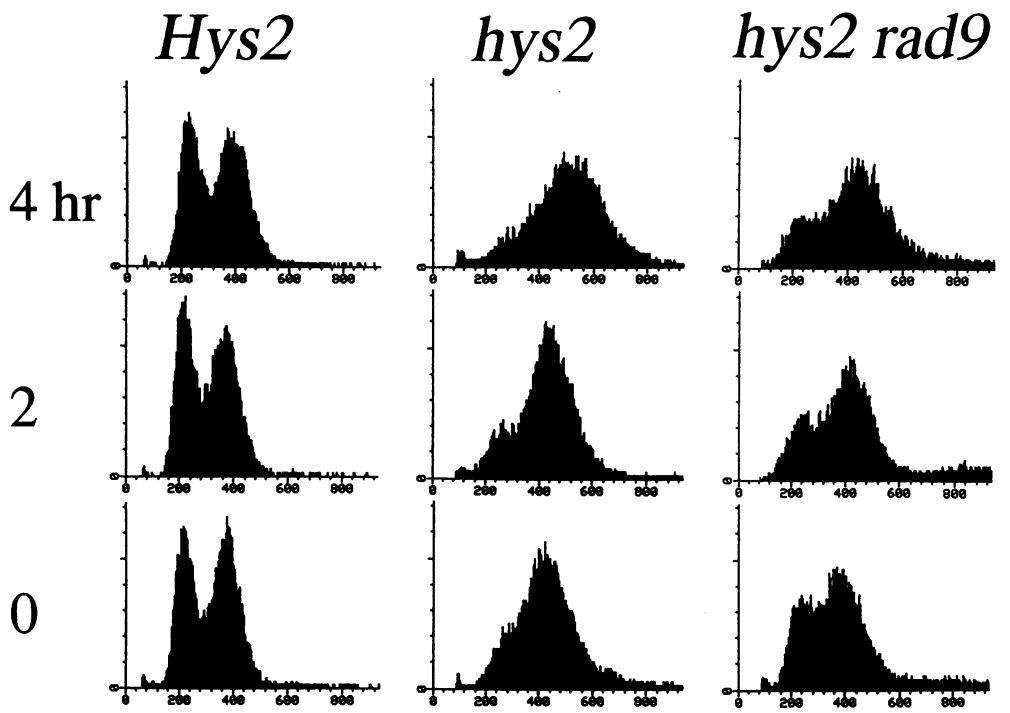

Figure 2. DNA flow cytometry analysis of *hys2-1* and *hys2-1 rad9* mutant cells. Strains KSH106 (*HYS2*), KSH542 (*hys2-1*) and KSH687 (*hys2-1 rad9Δ::LEU2*) were grown to early log phase in YEPD at 25°C. After shifting to 37°C, samples of cells taken at 2 h intervals were fixed, stained with propidium iodine and examined by flow cytometry. Control experiment was done for *hys2-1* cells by treating with 20 μg/ml nocodazole at 25°C for 4 h and subsequently shifting to 37°C for 4 h. This experiment confirmed that *hys2-1* cells arrest with a 2N content of DNA at 37°C.

analysis is that the *hys2-1* mutation does not disrupt the bulk of DNA replication at the restrictive temperature. The *hys2-1* temperature sensitive mutation may be leaky as many leaky DNA synthesis mutants (e.g. *cdc17*) (33) that arrest with a G2/M content of DNA. It is noted that the culture of *hys2-1* mutant accumulated cells with a late S to G2/M phase DNA content at 25°C. The *hys2* mutant appears to have a defect in S-G2/M phase execution at the permissive temperature.

Although the bulk of DNA is replicated in the *hys2-1* mutant, there could be small amounts of unreplicated DNA sufficient to cause arrest of the cell cycle. To determine whether DNA is completely replicated in *hys2-1* cells, chromosomes of the arrested cells were analyzed by pulse field gel electrophoresis (Fig. 3). In this assay only fully replicated DNA enters the gel; incompletely replicated DNA (e.g. replication loops or single-stranded regions) causes DNA to be retarded in wells (28). DNA was prepared from the *hys2-1* strain that had been incubated at the restrictive temperature for 4 h. As controls, the *hys2-1* strain was treated with α-factor mating pheromone (blocked in G1 phase), HU (blocked in S phase) or nocodazole (blocked in M phase) at the permissive temperature before DNA isolation. As shown previously (28), DNA isolated from the HU-treated culture mostly remained in the wells after electrophoresis, whereas the majority of DNA from the mating pheromone or nocodazole treated cultures migrated into the gel. Compared with DNA isolated from the wild type cells, DNA prepared from *hys2-1* cells at the restrictive temperature entered the gel with greatly reduced efficiency (Fig. 3). Thus, it appears that DNA becomes incompletely replicated in *hys2-1* cells at the restrictive temperature.

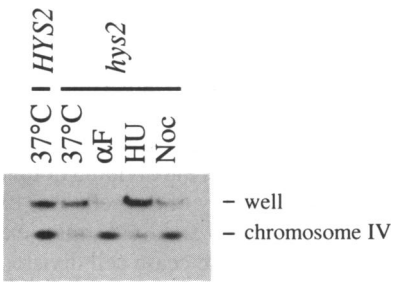

Figure 3. Chromosome abnormalities in the *hys2-1* mutant. Exponentially growing cultures of *hys2-1* (KSH542) and *HYS2* (KSH106) cells at 25°C were shifted to 37°C and incubated for 4 h in YEPD. As controls, *hys2-1* cells were incubated at 25°C in YEPD containing 10 μg/ml α-factor for 2.5 h, 10 mg/ml HU for 4 h and 20 μg/ml nocodazole for 4 h. The chromosomes of each sample were separated by pulse field gel electrophoresis, blotted and probed with a DNA fragment of the *TRP1* gene which resides on chromosome IV. The top row of bands indicates where the samples were loaded (well) and contains residual material that was not able to migrate into the gel.

Therefore, the most likely explanation for the phenotype of the *hys2-1* mutant is that it is defective in DNA replication, but somewhat leaky, so that DNA replication, while not complete, can result in the accumulation of DNA to a level approaching 2N.

If *hys2-1* mutants undergo cell cycle arrest because of DNA lesions, the arrest may be mediated by the product of the *RAD9* gene (24,34). To test the role of the *RAD9* checkpoint in cell cycle arrest in the *hys2-1* mutant, we constructed a *hys2-1 rad9Δ::LEU2*

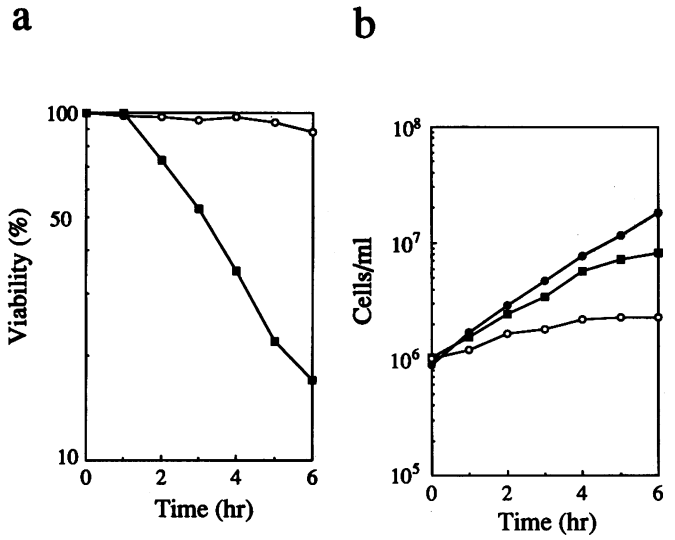

Figure 4. Viability of the *hys2-1 rad9* double mutant at the restrictive temperature. Strains KSH542 (*hys2-1*), KSH687 (*hys2-1 rad9Δ::LEU2*) and a wild type strain KSH106 were grown exponentially at 25°C and then shifted to 37°C. Cell viability was determined after shifting as described in Materials and Methods and the percent viability at intervals is shown (**a**). Samples were also analyzed for cell number (**b**). Symbols: *hys2-1*(○), *hys2-1 rad9 Δ::LEU2* (■), wild type (●).

double mutant strain. Cultures growing exponentially at 25°C were shifted to 37°C and the cell cycle properties were examined. Whereas the *hys2-1 RAD9*$^+$ strain exhibited G2/M arrest when incubated at 37°C for 4 h, introduction of the *rad9Δ::LEU2* mutation in *hys2-1* reduced the level of arrest at G2/M (Fig. 2). Thus, the *rad9* mutation appears to largely alleviate the cell cycle arrest in the *hys2-1* mutant. If this failure to arrest is caused by the removal of a checkpoint, one would expect to observe a concomitant loss of viability in the dividing double mutant strain (24,35). Indeed, when viability of the cells was examined in the same experiment, *hys2-1 rad9Δ::LEU2* strains exhibited a rapid loss of viability and a failure to cease cell division at 37°C (Fig. 4). We conclude that the cell cycle arrest of *hys2-1* after shift to the restrictive temperature requires an intact *RAD9* gene.

Cloning of the *HYS2* gene

We cloned the *HYS2* gene by complementation of the HU sensitive phenotype of a *hys2-1* mutant. After transforming a genomic library constructed on YCp50 into strain KSH542 (*hys2-1 ura3*), we screened for transformants able to grow on YEPD containing HU. A single plasmid-linked transformant was identified and the transformant contained a plasmid (p12) with a 10 kb insert. Analysis of the subclones of the p12 insert demonstrated that the 2.5 kb *Hin*dIII-*Sal*I fragment complemented both HU sensitivity and temperature sensitive growth (data not shown). We found that the *HYS2* gene maps to chromosome X by using the cloned gene to probe a blot of yeast chromosomes separated by pulse field gel electrophoresis (data not shown). The nucleotide sequence of the 2.5 kb *Hin*dIII–*Sal*I DNA fragment on both strands was determined (DDBJ accession no. D50324). A single open-reading frame was found within this region, corresponding to the *HYS2* gene. This sequence predicts a protein of 487 amino acids, assuming that translation begins at the first ATG. A search through several sequence banks failed to identify any known proteins with significant sequence similarity to Hys2. The nucleotide sequence at the 3′-region of the *HYS2* gene is identical to the upstream sequence of the *SUI2* gene (36), indicating that these genes reside adjacent to each other. This finding is consistent with our result of chromosome blotting of *HYS2*, because the *SUI2* gene is located near the centromere on chromosome X. Any genes have not been genetically mapped close to *SUI2*, that correspond to the *HYS2* gene.

To determine if *HYS2* is essential for viability in *S.cerevisiae*, we created a disruption in the *HYS2* open reading frame. A *LEU2* fragment was replaced by the 1 kb *Bam*HI fragment internal to the *HYS2* coding region and the *Xba*I-*Pvu*II fragment containing the *LEU2* insert was used to transform a diploid strain KSH172. Southern blot analysis of Leu$^+$ transformants showed that one of two copies of the *HYS2* gene had been disrupted. When this diploid (KSH700) was sporulated, tetrads segregated 2:2 viable:non-viable. All the viable spores were Leu$^-$, indicating that they had the wild type copy of the *HYS2* gene. This result is consistent with the *HYS2* gene product being essential for growth. Microscopic examination of the inviable spores indicated that they germinated to form microcolonies of three to 11 cell bodies. The *HYS2/hys2Δ::LEU2* diploid (KSH700) was transformed with a plasmid YCp33-HYS2 bearing the *HYS2* and *URA3* genes and after sporulation, tetrad analysis was carried out. In every ascus, two to four spores were viable and every Leu$^+$ spore clone carrying the *hys2Δ::LEU2* mutation was Ura$^+$. Even after culturing the Leu$^+$ Ura$^+$ spore clones for several generations in non-selective YEPD medium, no Leu$^+$ Ura$^-$ segregants were selected. Thus, the *HYS2* gene appears to be essential for cell growth.

The nucleotide sequence of the *HYS2* 5′-noncoding region revealed that a single *Mlu*I site is located 107 bp upstream from the translational initiation site of *HYS2*. The sequence 'ACGCGT' or a closely related sequence, called the *Mlu*I cell cycle box (MCB) element, is required for the coordinate expression of a group of DNA replication genes including *CDC17* (DNA polymerase α), *CDC2* (DNA polymerase δ), *POL2* (DNA polymerase ε), *CDC9* (DNA ligase) and *CDC8* (thymidylate kinase), beginning late in G1 and extending into S phase (reviewed in ref. 37). We analyzed a *HYS2* gene transcript by Northern blotting using the methods described in Materials and Methods. We identified a single transcript of ~1.7 kb in exponentially growing cells (data not shown). To determine whether the expression of *HYS2* is coordinately regulated throughout the cell cycle, *HYS2* mRNA levels were monitored in a culture synchronized in G1 phase by treatment with α-factor mating pheromone, released from the arrest by incubation in fresh medium and then sampled at intervals of 20 min. As shown in Figure 5, accumulation of *HYS2* mRNA oscillated peaking around the time of bud emergence, which corresponds to late G1 or early S. However, the cell cycle regulation is modest (activated 2- to 3-fold) and the transcripts are present throughout the cell cycle.

DISCUSSION

We have screened for mutants that are sensitive to HU and identified four distinct *HYS* genes. The *hys* mutations are expected to fall into two classes. The first class consists of mutations defective in coupling mitosis to the completion of DNA replication. In the presence of HU, they enter into mitosis with incomplete chromo-

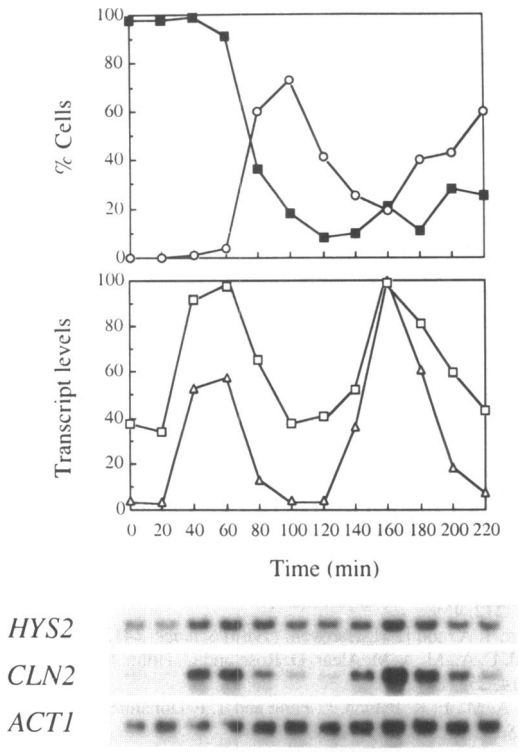

Figure 5. Northern blot analysis of *HYS2* transcription. Three micrograms of total RNA prepared from samples of cells taken at 20 min intervals after α-factor release were separated on 1% formaldehyde-agarose gels and analyzed by Northern blot analysis using the *HYS2* probe. The cell cycle synchronization was confirmed by probing with *CLN2*. The levels of the *CLN2* transcript fluctuate during the cell cycle, peaking at the G1/S boundary (29,44). The same blot was hybridized with *ACT1* as loading control (bottom panel). Samples were also analyzed for the cell-cycle synchrony (top panel). Unbudded cells (■) and cells with small buds (○) were quantitated. The intensity of *HYS2* and *ACT1* bands was quantitated by densitometric tracing with a film of an appropriate exposure and levels of *HYS2* (□) and *CLN2* (Δ) mRNAs were normalized relative to *ACT1* transcript levels (middle panel). The peak value of transcript levels was given a value of 100 for graphical purpose.

somal duplication, resulting in lethality. Enoch *et al.* (16) have used this screen in fission yeast to identify genes involved in checkpoint control that prevents mitosis from occurring until S phase is completed. DNA sequence and complementation analyses revealed that *HYS1* is identical to *SPK1/MEC2/RAD53/SAD1*, a gene already known to be involved in the S phase checkpoint (38–41). Thus, the *hys1-1* mutation is assigned to the first class. The second class consists of mutations defective in DNA replication itself. Because HU blocks DNA synthesis, mutations defective in DNA metabolism are predicted to cause more severe growth retardation in the presence of HU. In contrast to the first class, these mutations do not advance the cell cycle in the presence of HU. We have shown the evidence indicating that the *hys2* mutation is assigned to the second class. The screen is unlikely to be saturated, as we identified only one allele of each *HYS* gene.

A single recessive mutation in *HYS2* is responsible for both HU sensitive and temperature sensitive growth phenotypes. We have shown that *HYS2* is an essential gene located on chromosome X adjacent to *SUI2*. Although the molecular function of *HYS2* is yet unknown, several observations suggest that Hys2 is likely to be involved in DNA replication. At the restrictive temperature, the *hys2-1* mutation causes an accumulation of cells with a large bud in which a single nucleus is found at the neck of the bud. The average DNA content in a population of *hys2-1* cells is shifted toward the G2 value. These two phenotypes indicate that while the bulk of DNA replication is completed in *hys2-1* cells at the restrictive temperature, mitosis is blocked. The *hys2-1* mutation also causes elevated levels of both chromosome loss and genetic recombination and causes an increase probability of death in cells lacking a functional *RAD9* checkpoint gene.

The *hys2-1* mutation causes cells to arrest with DNA replication essentially being complete at the level of detection provided by the flow cytometry. The *hys2-1* mutant arrested in the G2 phase at the restrictive temperature, whereas the *hys2-1 rad9* double mutant failed to arrest and died rapidly. This is consistent with the previous observation that the RAD9 checkpoint is required for the late S/G2 phase arrest of mutants defective in DNA replication (24). It has been suggested that DNA lesions activate the *RAD9* checkpoint to arrest cell division in G2 prior to mitosis. DNA damage is evident in *hys2-1* cells at the level of elevated mitotic recombination (32) and the inability of chromosomes to migrate on pulse field gel electrophoresis may be associated with the DNA damage. Therefore, it is possible that DNA lesions formed at the restrictive temperature in the *hys2* mutant activate the *RAD9* checkpoint to arrest in G2. Finally, it should be noted that cell cycle arrest in *cdc8* mutants at the restrictive temperature and in HU treated cells does not require the *RAD9* gene (24,42,43). The *CDC8* gene encodes thymidylate kinase required for dTTP synthesis and HU is a potent inhibitor of ribonucleotide reductase necessary for deoxyribonucleotides production. Thus, limitation of the precursors for DNA synthesis does not appear to result in *RAD9* checkpoint activation. This may suggest that *HYS2* plays a role in a step of DNA replication besides production of precursors for DNA synthesis.

Most of the genes involved in DNA replication periodically expressed during the cell cycle, peaking at the G1/S boundary. This periodic expression is dependent on the MCB element located in their promoter regions (37). The *HYS2* gene has a copy of the MCB element at an appropriate distance upstream of the translation start site. However, the cell cycle periodicity of *HYS2* transcription is rather weak (varied only 2- to 3-fold) and the transcript is present throughout the cell cycle. Furthermore, cells expressing *HYS2* constitutively from the *GAL1* promoter did progress through the cell cycle without any observable perturbation (data not shown). These results suggest that the transcriptional control during the cell cycle is not critical for roles of *HYS2*. Additionally, these results may imply that the Hys2 protein is present throughout the cell cycle and has other roles in DNA metabolism besides DNA replication, such as DNA damage repair. In fact, the *hys2-1* mutation conferred sensitivity to the DNA damage-inducing agent MMS.

In summary, characterization of *hys2* mutant demonstrated that screening of *hys* mutants is useful for better understanding cell cycle regulation in S and G2 phases. In addition to more extensive studies of *HYS1/SPK1/MEC2/RAD53/SAD1* and *HYS2*, characterization of *hys3* and *hys4* mutants will further our understanding replication control during cell cycle.

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